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*SULFOLIPID FROM VIRULENT TUBERCLE BACILLI**

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It is known that the bacterial cells of pathogenic human and bovine varieties of *M. tuberculosis* fix the dye neutral red in its salt form (red) to their surface in an alkaline aqueous environment, while mutant strains of these organisms which do not manifest this behavior are highly attenuated or avirulent.[1, 2] This is a preliminary report of investigations providing evidence that the material responsible for this fixation of neutral red is a new type of sulfolipid.

Surface cultures of living bacterial cells from a synthetic medium containing ammonia as the sole source of nitrogen, malic acid (0.3%), glucose (2%), glycerol (0.5%), and the usual inorganic salts, were washed with distilled water after 14 or 28 days of growth. They were briefly extracted, moist, with hexane (Skellysolve B) to which was added some decylamine (0.05%). The extracts were evaporated on a steam bath under N_2 to a small volume and shaken with aqueous 1 *N* citric or 0.1 *N* hydrochloric acid in order to remove most of the decylamine. Such crude extracts of the fully pathogenic strains, H37Rv (human) or Vallée (bovine), when shaken with a dilute aqueous solution of neutral red hydrochloride, became brightly colored red. The neutral red dye is "pulled" into the hexane to form a hexane-soluble salt with the acidic substance or substances dissolved in the hexane; and the amount of neutral red so fixed was measured by appropriate spectrophotometric methods.

Analysis of this reaction led to the conclusion that the material responsible for this "fixation" of neutral red was strongly acidic, and it was assumed at first to be a phospholipid. However, as the work progressed, the phosphorus content of the most abundant neutral red fixing fraction of the crude extracts of virulent strains, separated by chromatography on silicic acid-celite and silica gel or silica gel-celite, decreased strikingly. A test for sulfur revealed its presence in significant amounts. Further purification, including acetone precipitations to eliminate more completely any contamination with phospholipid, has yielded a slightly amber-colored, strongly acid-fast lipid, oily above 25°C, containing 1.1 per cent sulfur and less than 0.02 per cent phosphorus, and fixing 85 mcg of neutral red per mg. This is consistent with about one mole of neutral red fixed in salt form per atom of sulfur, and with the acid equivalent weight of the material, about 3,000. This fraction constitutes 0.1 to 0.2 per cent of the dry weight of the bacterial cells of the strain H37Rv.

Infrared spectrophotometric (IR) and nuclear magnetic resonance (NMR) analyses have been made of this and other similar fractions for comparative purposes, with the generous collaboration of Dr. John C. Craig and Dr. Evan Horning of the Laboratory of the Chemistry of Natural Products, National Heart Institute, National Institutes of Health. In Figure 1 are shown the IR and the NMR curves given by this material. These studies have shown the following:

(1) The material contains a peculiarly high proportion of methyl groups suggestive of the methyl-branched-chain fatty acids described by several previous investigators in hydrolyzed preparations of the acetone soluble lipids of tubercle bacilli.[3, 4, 5]

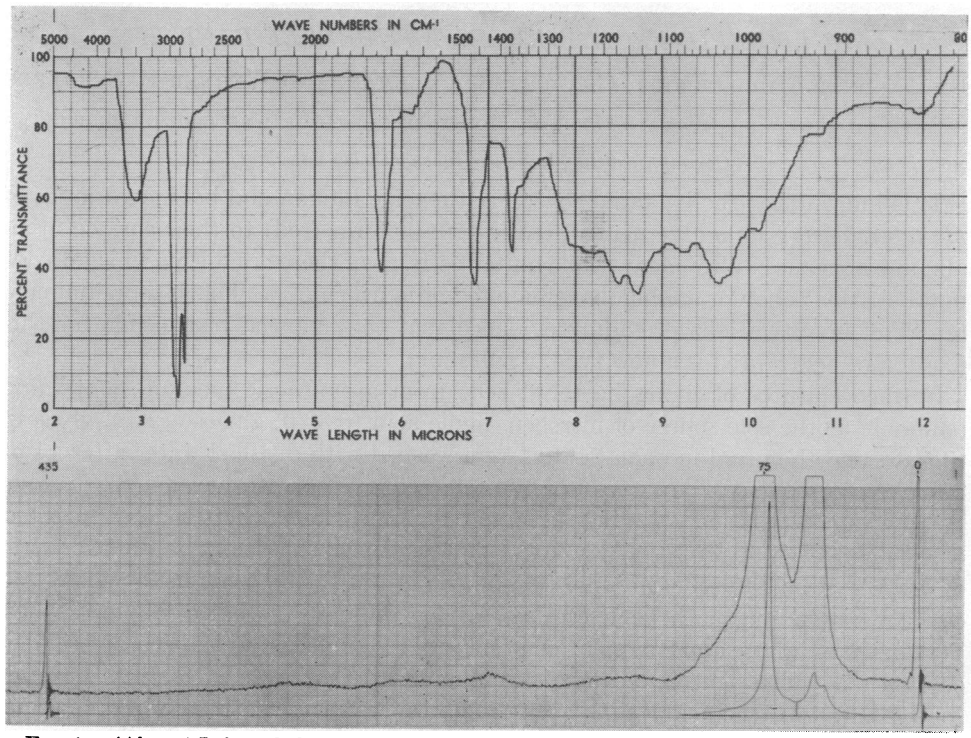

FIG. 1.—(Above) Infrared absorption spectrograph of non-polar sulfolipid fraction from H37Rv, 10% solution in CCl_4, on Model KM-1, Baird-Atomic, Inc., spectrophotometer. (Below) Nuclear magnetic resonance spectrograph of non-polar sulfolipid fraction from H37Rv, in $CDCl_3$, on 60 KC instrument at Varian Associates, Palo Alto, Calif., courtesy of Dr. James N. Shoolery.

(2) It contains a very small proportion of alcoholic hydroxyl groups, which is consistent with its insolubility in water and in methanol, and its high solubility in mineral oil, petroleum ether, chloroform, diethyl ether, and acetone.

(3) The IR absorption bands at 1020–1060 cm^{-1} and 1140–1200 cm^{-1} are consistent with those of a sulfonic acid.

(4) It contains no carbon to carbon unsaturated bonds, which excludes the presence of the phthienoic type of fatty acid.[6, 7]

(5) It contains a prominent carboxylic acid ester absorption band (1,740 cm^{-1}) and little or no free carboxylic acid (1,720 cm^{-1}).

Cells of the H37Rv strain were cultivated on medium containing 1 millicurie of $S^{35}O_4$ per liter. Crude extracts were prepared as described above. They proved

to contain significant amounts of S^{35}, and chromatographic separation of such extracts into various fractions revealed good parallelism between radioactivity and neutral red fixing activity (see Table 1). These results indicate that the prin-

TABLE 1

CHROMATOGRAPHIC ANALYSIS OF CRUDE MATERIAL EXTRACTED BY HEXANE-DECYLAMINE FROM H37Rv ON SILICIC ACID[a]: CELITE (200 MG:100 MG PER MG OF CRUDE MATERIAL), AT 25C; 2 COLUMN VOLUMES OF EACH ELUENT

Eluting Solution	Experiment 1			Experiment 2		
	mg	mcg NR[c]	cpm[d]	mg	mcg NR[c]	cpm[d]
	180	8.2	1,300	84	12.1	1310
Chloroform[b]	120	<0.5	<30	58	<0.5	<30
Chloroform + 4% methanol	32.5	28.5	3,570	12.4	31.0	3,680
Chloroform + 10% methanol	11.6	44.0	3,820	14.5	34.0	3,830
Methanol,[e] 100%	1.4	15.5	715	3.5	38.0	825
Recovery, %	*92*	*97*	*70*	*105*	*99*	*95*

[a] Containing 7.2% H_2O.
[b] Containing 0.75% ethanol.
[c] Expressed as mcg neutral red base fixed per mg of material in hexane.
[d] Expressed as counts per mg per min under standardized conditions, S^{35}.
[e] Most of the neutral red fixing material in this fraction is insoluble in acetone and contains significant amounts of phosphorus.

cipal neutral red fixing material from this strain constitutes either a single molecular species exhibiting much tailing on chromatography or, more likely, a group of closely related sulfolipids with slight differences in polarity. These studies have also revealed that more or less neutral red fixing material of high polarity, soluble in methanol and having low radioactivity, can also be extracted from this and other strains of tubercle bacilli, regardless of their biologic properties or the ability of the whole bacterial cells to fix neutral red in an alkaline aqueous environment. These fractions contained up to 4.2 per cent phosphorus, and their amounts in hexane-decylamine extracts varied in particular with the duration of the extraction period and the age of the cultures, being especially prominent in extracts of old autolyzing populations.

Comparative studies have also been made of other selected strains of *M. tuberculosis* (see Table 2).

TABLE 2

CHARACTERISTICS OF CULTURES AND MATERIAL EXTRACTED FROM FIVE DIFFERENT STRAINS OF TUBERCLE BACILLI

Strains	Cord Formation	Neutral Red Reaction, Whole Cells	Crude Material Extracted with Hexane-decylamine, mg[a]	Neutral Red Fixing Activity per mg of Crude Material, mcg NR[b]	Radioactivity per mg of Crude Material, S^{35}
H37Rv	Strong	Strong	12–17	8–15	1,300 cpm[c]
Vallée	Strong	Strong	12–17	8–15	(Not done)
BCG-P	Strong	Strong	12–17	5–10	120 cpm
BCG-T	Weak	Weak	10–15	0–2	(Not done)
H37Ra	Absent	Absent	10–15	0–2	48 cpm

[a] Hexane-soluble material per gram dry weight of living bacterial cells.
[b] Expressed as mcg of neutral red base fixed per mg of material in hexane.
[c] Expressed as counts per mg per min under standardized conditions.

Extracts have been prepared from two BCG strains of *M. tuberculosis* which are partially attenuated in pathogenicity. One, the least attenuated, fixes neutral red well as whole bacterial cells, and hexane-decylamine extracts of this strain fix at least half as much neutral red as the fully pathogenic strains. However, the sulfur content of the neutral red fixing fractions from this strain is only one-tenth

that of the H37Rv strain; and it appears to produce little or none of the relatively nonpolar neutral red fixing material synthesized by and so easily extracted from the surface of the two fully pathogenic strains. Most of the neutral red fixing activity of the whole bacterial cells and of the extracts of this attenuated strain is fairly attributable to phospholipid.

Cells of the other, more attenuated, strain of BCG fixed neutral red very weakly and yielded crude extract material with one-sixth as much neutral red fixing activity as the less attenuated strain.

In all experiments with the nonpathogenic strain H37Ra, the whole cells of which do not fix neutral red, little or no neutral red fixing material was released into hexane-decylamine extracts, and such an extract contained very little sulfur.

Preliminary tests of the least polar sulfolipid fraction from the strain H37Rv, dissolved in light paraffin oil (Bayol F), and injected by the intracutaneous route into guinea pigs, have shown that it has significant local toxicity both for normal and for tuberculous animals.

Finally, the oily character of this sulfolipid material as well as its apparently superficial location in the bacterial cells would seem eminently to suit the requirements of a capsular or envelope substance responsible for the morphologic serpentine cord formation so characteristic of cultures of neutral red fixing, pathogenic strains of tubercle bacilli.[8] Therefore, it seems reasonable to postulate that this material plays a prominent role in the biology of tuberculosis.

Summary.—Evidence has been presented that the principal material responsible for the fixation of neutral red by fully pathogenic strains of tubercle bacilli consists of sulfolipid. Physical and chemical studies of this material indicate that it is a kind of chemical substance heretofore unrecognized in biological products.

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